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Differential Regulation of the Nodulation Zone by Silver Ions, L-α-(2-Amino-Ethoxyvinyl)-Glycine, and the *skl* Mutation in *Medicago truncatula*

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Nodule formation in *Rhizobium*-legume symbiosis is negatively regulated by ethylene. Ethylene inhibitors such as L-α-(2-amino-ethoxyvinyl)-glycine (AVG) and silver ions (Ag^+), the ethylene-insensitive *sickle* mutant, and transgenic plants were used to study ethylene-mediated responses in nodulation. The mode of action of ethylene inhibitors AVG and Ag^+, and the *skl* mutation occur at different steps in ethylene biosynthesis and perception. Their effects on root growth and nodulation phenotypes, in particular nodule distribution along the primary root, were compared in this study. Ag^+ and AVG treatments showed similar root growth responses to *skl* mutant. However, nodule distribution in the hypernodulating *skl* mutant is different from that of wild-type plants grown on either AVG or Ag^+. AVG increased nodule numbers and widened the nodulation zone, while the *skl* mutant had an increased number of nodules within the susceptible zone of nodulation. Ag^+ reduced nodule numbers, restricted the nodulation zone, and restored the nodulation phenotype of *skl* to that of the wild type.

Key words: ethylene, nodulation, *Rhizobium*-legume symbiosis, *Medicago trancatula*

INTRODUCTION

Phytohormones are known to control nodule initiation and development in *Rhizobium*-legume symbiosis. Ethylene, a gasous phytohormone, negatively affects the process of nodule development (Hirsch & Fang 1994; Guinel & Geil 2002; Fergusson & Mathesius 2003). The involvement of ethylene in nodulation has been suggested for more than three decades (Hirsch & Fang 1994; Guinel & Geil 2002; Ferguson & Mathesius 2003). Later, the involvement of ethylene in nodulation was demonstrated using ethylene inhibitors such as AVG and Ag^+, using an ethylene-insensitive mutant, and transgenic plants.

The inhibitory effect of ethylene on nodulation could be overcome by the treatment with AVG (Peters & Crist-Estes 1989; Ligero *et al*. 1991; Lee & LaRue 1992), or Ag^+ (Caba *et al*. 1998; Nukui *et al*. 2000). AVG inhibits ethylene biosynthesis by inactivation of ACC synthase, an enzyme that converts S-adenosyl methionine to ACC (Lin *et al*. 2009). Ag^+ is thought to block ethylene perception by replacing the copper cofactor present in the ethylene-binding site of the receptor. Receptors containing Ag^+ bind ethylene but fail to transduce the signals from the receptors to their downstream signalling cascade (Rodriguez *et al*. 1999).

Also, the use of an ethylene-insensitive mutant of *Medicago truncatula* (the *skl* mutant), which has an increased number of nodules when inoculated with *S. meliloti* (Penmetsa & Cook 1997), confirms the negative role of ethylene in the nodule developmental program. The *skl* mutant has a mutation in a component of ethylene signalling, most likely similar to AtEIN2 (Penmetsa & Cook 1997). Another support for the role of ethylene in nodulation is shown in transgenic *L. japonicus* plants carrying the melon ethylene receptor gene *Cm-ERS1/H70A* that are defective in ethylene binding as a result of a point mutation at the 70th amino acid (Nukui *et al.* 2004). In this transgenic plant, nodule primordia and infection thread numbers are increased because of its ethylene insensitivity.

The mode of action of ethylene inhibitors AVG and Ag^+, and the *skl* mutation occur at different steps in ethylene biosynthesis and perception. However, none of previous studies directly compared their effects on root growth and nodulation phenotypes, in particular nodule distribution along the primary root following rhizobioal inoculation. This nodulation phenotype is an important feature to study the autoregulation of nodulation which controls the formation of nodule numbers in younger root tissues (Caetano-Anollés & Gresshoff 1991; van Noorden *et al*. 2006). In this report, the effect of the ethylene inhibitors Ag^+ and AVG on nodule distribution along the primary root was investigated and compared to that of the *skl* mutant. In addition, the ethylene inhibitor Ag^+ and AVG were used to test whether Ag^+- or AVG-treated wild-type plants would phenocopy the nodulation and root phenotypes of *skl* upon inoculation with rhizobia such as

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the increased nodule numbers, and a reduction of root growth. Likewise, AVG was used to test whether it induced similar nodule and root growth responses as Ag^+ in the *skl* mutant.

MATERIALS AND METHODS

Plant and Bacterial Growth Conditions. Seeds of *skl* mutant were obtained from Prof. Douglas R. Cook (Penmetsa & Cook 1997). Seeds of cv Jemalong A17 were used as the wild type. Seeds were scarified and surface sterilized with 6.25% (v/v) sodium hypochlorite for 15 min. After several washing, seeds were incubated on nitrogen-free Fåhraeus agar medium (Fåhraeus 1957) in dark-cold condition (4 °C) for 2 days to break their dormancy. A drop of sterile water was applied on each seed to prevent the seeds from drying. Seeds were then germinated by incubating in the dark at 28 °C overnight, Seedlings with similar root length were selected and transferred to 15 cm Petri dishes containing Fåhraeus agar medium. The seedlings were incubated vertically in the growth chamber with photon flux density of 90 µmol m^{-2} s^{-1}, and 16 h of light per day at 20 °C for 2 days. To reduce light intensity around roots, pieces of dark-thick paper were placed at the lower half between the plates. After 2 days incubation, the seedlings were transferred to fresh Fåhraeus plates containing AVG and/or Ag^+, and incubated in the same growth chamber. The seedlings were flood-inoculated at the root tips with 5 µl of diluted *Sinorhizobium* suspension 24 h later. The positions of the root tips at the time of inoculation (RT_0) were marked on the plates to provide an initial point of measurement for root growth and nodule position.

Sinorhizobium meliloti strain 1021 was grown in liquid Bergensen's modified medium (Rolfe *et al*. 1980) at 28 °C overnight, and diluted with sterile water to an optical density (OD600) of 0.1 or approximately 10^7 cells/ml. As controls, roots were inoculated with an equivalent amount of diluted Bergensen's modified medium.

RESULTS

Ag^+ Restores the Wild-Type Nodule Phenotype in *skl*. After transferring seedlings to medium containing Ag^+, newly grown wild-type roots were thinner with a smaller root diameter (Figure 1a). These morphological changes were not observed on the wild-type plants transferred to control plates devoid of Ag^+. The plants continued forming thinner roots after inoculation with *S. meliloti*. Fresh root sections of the region 15 to 20 mm below RT_0 showed that the reduced root diameter was a result of a decrease in cell expansion, consistent with the role of ethylene on radial cell expansion (Figure 1b compared to 1c). The inoculated *skl* plants, regardless of Ag^+ treatments, also had thinner roots compared to the control wild-type plants (Table 1).

In inoculated wild-type plants, the increase of Ag^+ concentration to 0.1 µM gradually enhanced the primary root growth (Figure 1d). In inoculated *skl* plants, 0.01 µM Ag^+ had no effect on primary root growth, but treatment of 0.1 µM Ag^+ sharply increased the primary root growth to almost double that of control treatment (197%) (Figure 1d). In contrast to its primary root growth response, nodule numbers of wild-type plants were not affected by 0.01 and 0.1 µM Ag^+, yet at a higher Ag^+ concentration (1 µM), nodule numbers were significantly reduced (LSD test, á=0.05) (Figure 1e). Similarly, in *skl* plants, nodule numbers were not affected by a low concentration of Ag^+ (0.01 µM). Higher Ag^+ concentrations (0.1 and 1 µM), however, sharply reduced nodule numbers in *skl* plants to the level of the corresponding wild-type plants (Figure 1e).

Nodule distribution in *skl* plants grown on medium containing Ag^+ were then analysed and compared with control wild-type and *skl* plants (devoid of Ag^+). Nodules were formed on wild-type roots within the region close to RT_0 (Figure 1f). In *skl* plants, an increased number of nodules were formed in the susceptible zone of nodulation, producing a cluster of nodules with reduced size (Figure 1g). The same nodulation phenotype was also observed in *skl* plants grown on medium containing 0.01 µM Ag^+ (Figure 1h). In contrast, *skl* plants grown on 0.1 and 1 µM Ag^+ had significantly reduced nodule numbers (Figure 1e), with nodules formed only in the narrow zone close to RT_0 (Figure 1i). In addition, the nodule size was also comparable to that of the wild-type plants (Figure 1f and i). Therefore, Ag^+ concentrations of 0.1 and 1 µM restored the wild-type nodule phenotype in *skl* plants.

The events of Ag^+-enhancement of primary root growth and Ag^+-inhibition of nodulation in *skl* plants occurred at the same Ag^+ concentration (0.1 µM) (Figure 1d and e). A significant correlation between primary root growth and nodulation was observed in Ag^+-treated *skl* plants ($R = -0.814$; $P < 0.001$), but not in Ag^+-treated wild-type plants ($R = -0.447$; $P = 0.133$). Taken together, the hypernodulation phenotype of *skl* caused root growth inhibition, and when the nodulation phenotype was restored to the wild-type level, the inoculation-reduced root growth was not present.

AVG Increases Nodulation by Increasing the Susceptible Zone of Nodulation. As shown in Figure 2a, AVG concentration determined the response of primary root growth. In inoculated wild-type plants, for example, 0.1 µM AVG increased primary root growth, while 10 µM AVG appeared toxic to the plant and severely reduced primary root growth. Similarly, 10 µM AVG inhibited primary root growth in inoculated *skl* plants. AVG also reduced the root diameter of inoculated wild-type plants in a similar fashion to Ag^+ treatment (as shown in Figure 1a-c).

AVG increased nodule numbers of wild-type roots in a dose dependent manner, with 0.1 and 1 µM AVG giving the highest result (Figure 2b). These AVG treatments induced approximately three times the number of nodules developed in the untreated control wild-type roots, but with smaller size. Interestingly, 0.1 and 1 µM AVG induced nodule formation further down toward the root tips (Figure 2c), indicating that AVG increases nodule numbers in wild-type roots by expanding the nodulation zone. Nodules were spread along the primary root to at least 70 mm from RT_0. In terms of nodule distribution along the root, AVG-treated wild-type plants had a distinct nodule phenotype

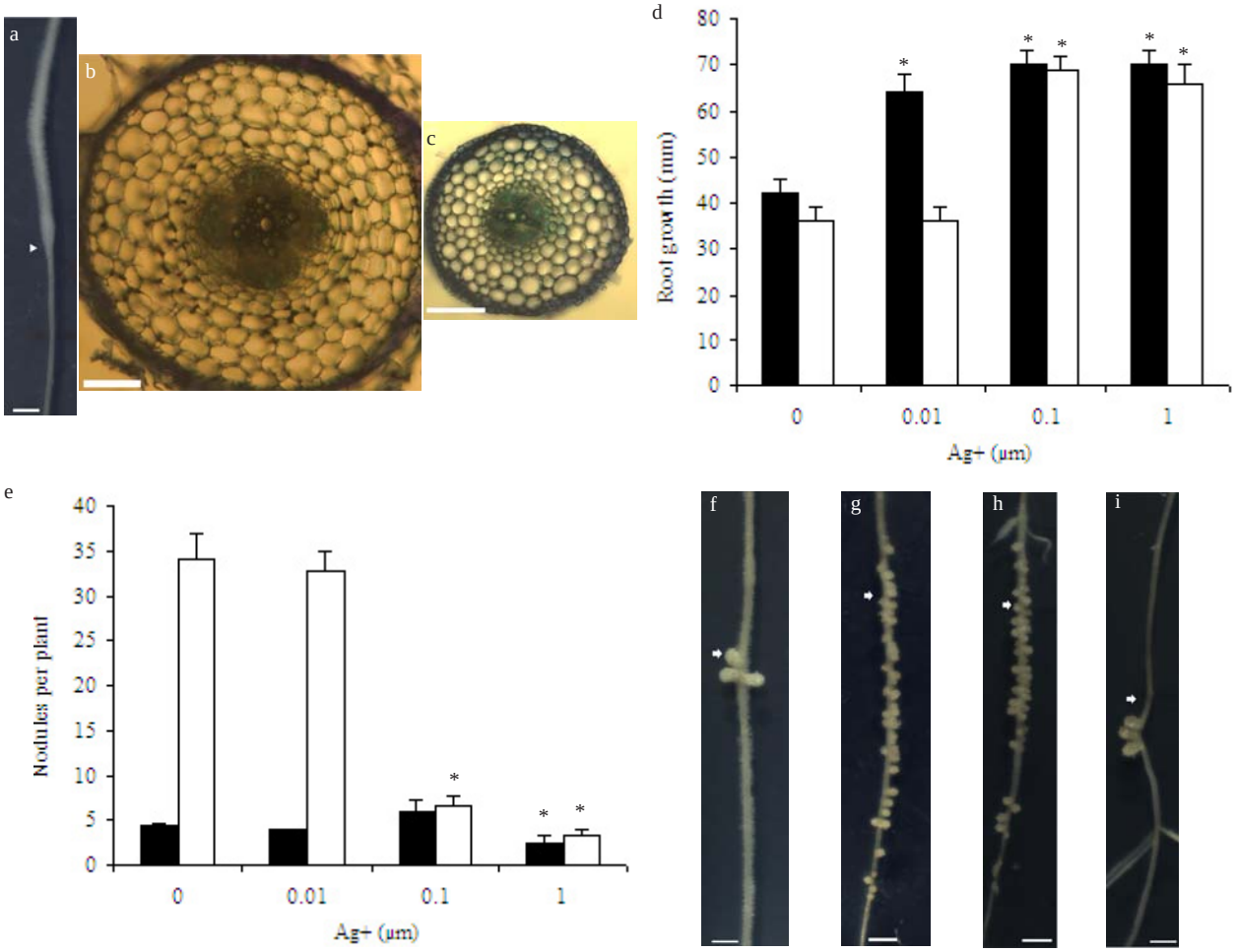

Figure 1. Root and nodulation responses of A17 (wild type) and *skl* mutant to Ag^+ treatments upon inoculation with *S. meliloti* strain 1021. (a) morphological changes of wild-type root after being exposed to Ag^+ (indicated by white arrow head). (b-c) comparison of root diameter of untreated (b), or Ag^+-treated (c) wild-type roots in the region 15-20 mm below RT_0. (d) primary root growth at 9 dpi. Values are the mean ± SE of 15 plants. (e) nodule numbers of wild-type and *skl* plants at various Ag^+ concentrations scored at 21 dpi. Values are the mean ± SE of 15 plants. (f-i) nodule phenotype of (f) wild type, (g) *skl*, (h) *skl* treated with 0.01 µM Ag^+, and (i) 0.1 µM Ag^+. White arrowheads indicate the position of RT_0. White bars = 2 mm (a, f-i), and 100 µm (b-c). Stars indicate significant differences to their corresponding control treatment (LSD test, á=0.05). ■: WT, □: *skl*.

Table 1. Effect of *Rhizobium* inoculation and Ag^+ on cortical cell length and root diameter of wild-type and *skl* plants

Treatment	Cortical cell length* (µm)	Root diameter# (µm)
A17 (wild type)	106 ± 7b	476 ± 50a
A17 + *Sm*1021	110 ± 8bc	421 ± 43ab
A17 + *Sm*1021 + Ag^+	109 ± 14b	370 ± 36bc
skl	121 ± 14c	358 ± 45bc
skl + *Sm*1021	72 ± 10a	340 ± 51c
skl + *Sm*1021 + Ag^+	102 ± 11b	374 ± 33bc

*Cortical cell length was determined from twenty cells selected randomly from the second and third cortical layers of the root in the region 15-20 mm below RT_0. Values are mean ± SD of ten plants. Values followed by the same letter do not differ significantly according to the Least Significant Difference (LSD) test, with the significance level (á)=0.05. #Root diameter was determined from cross-sections of fresh roots in the region 15-20 mm below RT_0. Values are mean ± SD of five plants. Values followed by the same letter in the column do not differ significantly according to LSD test (á = 0.05).

from the *skl* mutant, since the nodules did not form a cluster (Figure 1g compared to Figure 2c). In addition, increased nodule numbers in AVG-treated wild-type plants did not reduce primary root growth as observed in *skl* plants (Figure 2a-b). In contrast to its effect on wild type, 0.1 µM AVG had no effect on nodule numbers and nodulation zone of *skl* (Figure 2b). A higher AVG concentration (10 µM) decreased nodule numbers of *skl* plants, and the cluster of nodules in the susceptible zone of nodulation was maintained. Thus, when compared to Ag^+, AVG treatments had distinct effects on the nodulation response in both *skl* and wild-type plants.

The Negative and Positive Role of Ag^+ and AVG, Respectively, in the Regulation of the Nodulation Zone. As 0.1 µM Ag^+ restored the nodulation phenotype of the *skl* mutant to that of wild-type plants (Figure 1e and i), the same concentration of Ag^+ was used to test if it was able to restore the nodulation phenotype of AVG-treated wild type (0.1 µM) to untreated wild type. Ag^+ supplement had no detectable effect on nodule numbers in AVG-treated wild-type roots. For example, nodule numbers in AVG-treated and Ag^+-AVG-treated wild-type roots were 9.5 and 11.6, respectively (LSD test, $P_{0.05}$ = 0.184; Figure 3a). However, Ag^+ supplement narrowed the nodulation zone of AVG-treated wild-type plants, which was close to the

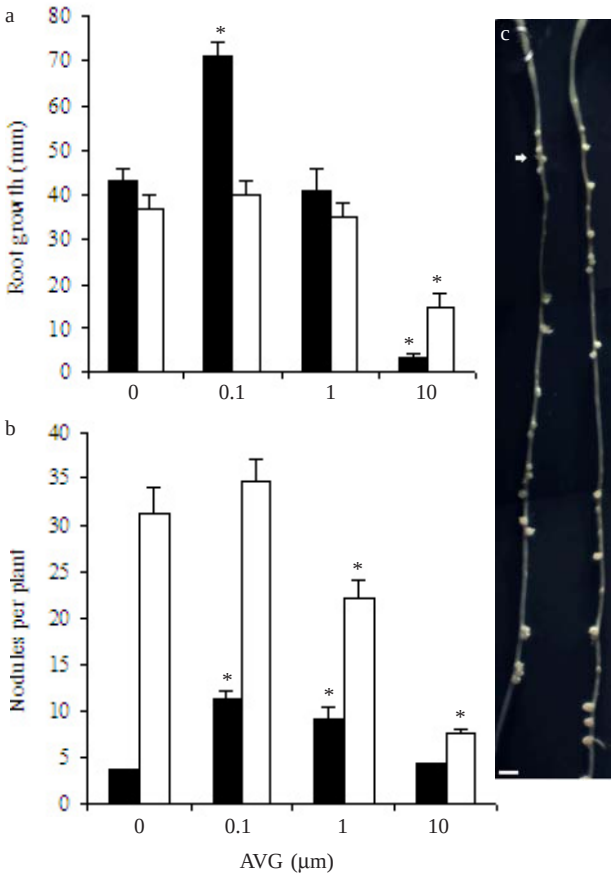

Figure 2. Root and nodulation responses of A17 (wild-type) and *skl* plants to AVG upon inoculation with *S. meliloti*. (a) Primary root growth measured at 9 dpi. (b) nodule numbers scored at 21 dpi. Values are mean ± SE of 15 plants. (c) nodule distribution in the wild-type root treated with 0.1 µM AVG. White arrowheads indicate the position of RT_0. Bar = 2 mm. Stars indicate significant values to their corresponding control treatment (LSD test, á = 0.01). ■: A17, □: *skl*.

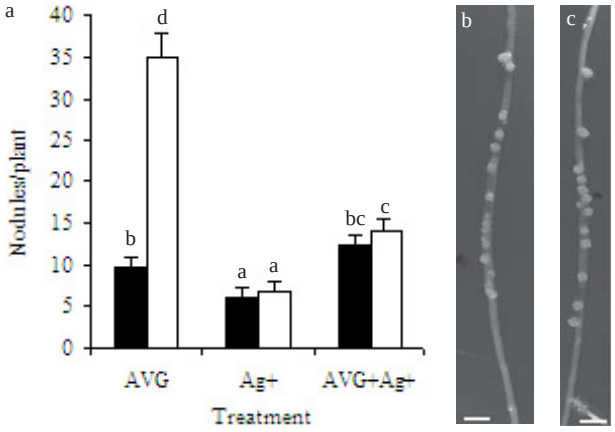

Figure 3. Effect of dual treatment of AVG and Ag^+ on nodule numbers. (a) nodule numbers of A17 (wild type) and *skl* in response to 0.1 µM AVG and 0.1 µM Ag^+. Treatments having the same lowercase letters are not significantly different according to the Least Significant Difference test (á = 0.05) (± SE; n=15). (b) nodule phenotype of AVG-Ag^+-treated A17 plants. (c) nodule phenotype of AVG-Ag^+-treated *skl* plants. White arrows indicate RT_0. Bars = 2 mm. ■: A17, □: *skl*.

RT_0 (Figure 3b). In *skl* plants, Ag^+ supplement significantly reduced nodule numbers in AVG-treated plants from 34.5 to 13.9 (Figure 3a), in which nodule distribution was also close to the RT_0 (Figure 3c). Nodule numbers of Ag^+-AVG-treated *skl* plants (13.9), however, were significantly higher than that of Ag^+-treated *skl* plants (5.5), but were comparable to that in Ag^+-AVG-treated wild-type plants (Figure 3a). In addition, the nodulation zone of Ag^+-AVG-treated *skl* plants was similar to that in Ag^+-AVG-treated wild type plants (Figure 3b-c).

DISCUSSION

AVG and Ag^+ Effect on Non-Symbiotic and Symbiotic Properties of *M. truncatula.* In this study, the root growth and nodulation responses of inoculated wild-type plants to the ethylene inhibitors AVG and Ag^+ have been examined and compared to that of the *skl* mutant. The results showed that in inoculated wild-type plants, the application of AVG stimulated root growth and reduced root diameter in a similar fashion to that of Ag^+ treatment or that of *skl* plants. This indicates that AVG and Ag^+ treatments, and the *skl* mutation give similar effects on non-symbiotic properties of *M. truncatula* upon inoculation with *Rhizobium*, which can be attributed to a defect or reduction in ethylene action on cell expansion (Figure 1). This is consistent with the role of ethylene in the root growth of Arabidopsis (Ecker 1995; Smalle & van der Straeten 1997).

In contrast to their effect on non-symbiotic properties, Ag^+, AVG and the *skl* mutation caused substantially different symbiotic properties upon inoculation, which are summarised in Figure 4. Ag^+ reduced nodule numbers in wild-type plants and restored the nodulation phenotype of the *skl* mutants to that of the wild-type plants (Figure 1e, i). In this study, Ag^+ concentrations between 0.01 and 0.1 µM were critical for nodule development in *skl*, because a sharp reduction of nodule number occurred between these two concentrations (Figure 1e). Previous reports have demonstrated the positive effect of Ag^+ treatment on nodulation in pea, alfalfa and *L. japonicus* (Lee & LaRue 1992; Caba *et al*. 1998; Nukui *et al*. 2000). In the present study, high Ag^+ concentration, up to 1 µM, did not induce any visible negative effect on other aspects of plant growth, thus excluding the possibilities that the toxic level of Ag^+ is the cause for the restoration of wild-type nodulation in *skl* plants. Fearn and LaRue (1991) reported that 0.1 µM Ag^+ decreased colony size of *Rhizobium*, suggesting that this concentration was inhibitory to bacterial growth. Results in this chapter showed that 0.1 µM Ag^+ did not decrease nodule numbers of wild-type plants. Therefore, the bacteria alone are not a likely cause for the reduction of nodule numbers in *skl*. Ag^+ is thought to inhibit ethylene perception by binding to ethylene receptors and then block the transmission of ethylene signals (Rodriguez *et al*. 1999). Ag^+ also reduced the expression of ethylene receptor genes in rice such as *OsERS1*, *OsERS2*, and *OsETR1* (Yau *et al*. 2004). However, the restoration of the nodule phenotype in *skl* by Ag^+ suggests that Ag^+ may not specifically block the ethylene

perception, because ethylene perception is altered in the *skl* mutant (Penmetsa & Cook 1997). Ag^+ has long been known as an inhibitor of ATPases (Knee 1992), enzymes involved in ion transport such as Na^+/K^+, Ca^{2+} and heavy metal ions (Axelsen & Palmgren 2001). Since the inhibition of ATPases would prevent normal cellular functions, Ag^+ might inhibit the specific and non-lethal ATPase function in nodulation.

AVG enhanced nodule numbers and widened the nodulation zone in wild-type plants (Figure 2b-c, 4). This is the first time that AVG has been shown to increase the nodulation zone in legumes, and raises the possibility that AVG can block the autoregulation mechanism in *M. truncatula*. An earlier report, however, showed that AVG increased nodule numbers in alfalfa (*M. sativa*) without the increase of susceptible nodulation zone (Peters & Crist-Estes 1989). Similarly, addition of AVG to *Vicia sativa* roots in a split root system using Jensen's liquid medium did not abolish autoregulation (van Brussel *et al*. 2002). This apparent contradiction might reflect differences in the species context or the experimental conditions.

Because AVG is an ethylene inhibitor, it would be expected then, that AVG treatment would give essentially the same result as the alteration in ethylene signalling, such as found in *skl*. The nodulation phenotype of AVG-treated wild-type plants, however, is different from that of *skl* mutants, as enhanced nodule numbers in *skl* plants are restricted to the susceptible zone of nodulation (Penmetsa & Cook 1997; Figure 1g). Thus, these conflicting results could not be reconciled as inhibition of ethylene signalling. In Arabidopsis, studies using ethylene inhibitors and the *ein2* mutant to determine the interaction of ethylene with abscisic acid (ABA) in root growth gave a similar paradox (Ghassemian *et al*. 2000). Similar results were also found in studies to determine the role of ethylene inhibitors on germination and seedling growth of barley (Locke *et al*. 2000). One possible explanation for this paradox is that AVG inhibits another pathway distinct from ethylene biosynthesis, which could be involved in the autoregulation mechanism. AVG inhibits ethylene biosynthesis by inactivation of ACC synthases through binding to their cofactor, pyridoxal 5'-phosphate (PLP) (Capitani *et al*. 1999). ACC synthases belong to the family of PLP-dependent enzymes (Alexander *et al*. 1994). Other PLP-dependent enzymes include ornithine decarboxylase (ODC) and arginine decarboxylase (ADC), the enzymes involved in the synthesis of polyamines such as putrescine, spermine and spermidine. AVG is also known as an inhibitor of cystathionine β–lyase, a PLP-dependent enzyme involved in methionine biosynthesis (Ravenel *et al*. 1998). It is possible that the increased area of nodule distribution by AVG is a result of the inactivation of a PLP-dependent enzyme other than ACC synthases. Further experiment would be necessary to test this hypothesis. Nevertheless, the nodule phenotype of *skl* exposed 0.1 μM AVG, the concentration that stimulated nodule numbers and widened the nodulation zone in wild type, was similar to untreated *skl* (Figure 1g), indicating that AVG action in widening the nodulation zone may require the SKL gene product. This could also be part of the pleiotrophic effects of the *skl* mutation in nodulation.

AVG	-	+	-	-	+	+	-	+
Ag^+	-	-	+	-	+	-	+	+
	A17	A17	A17	*skl*	A17	*skl*	*skl*	*skl*

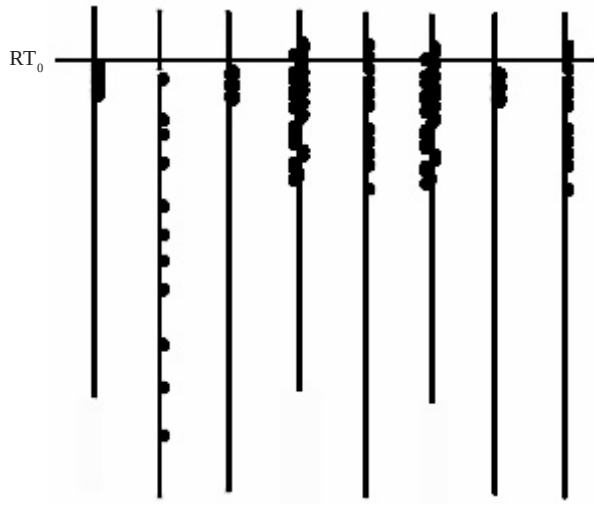

Figure 4. Summary of nodulation phenotypes in A17 (wild type) and *skl* treated with AVG and/or Ag^+. The table indicates combination treatments (marked with +) of AVG and Ag^+ in wild type and *skl*. The nodulation phenotype of each combination is shown below the column. Black dots represent nodules. The horizontal line represents RT_0.

Consistent with a single application of the ethylene inhibitor, Ag^+ supplement reduced the nodulation zone without affecting nodule numbers in AVG-treated wild type (Figure 3a-b), indicating the maintenance of nodulation homeostasis under these treatments. Conversely, AVG supplement increased nodule numbers and the nodulation zone of Ag^+-treated *skl* (Figure 3a, c). Together, results presented here suggest that AVG and Ag^+ have a positive and negative role, respectively, in the regulation of nodule numbers and nodulation zone in *M. truncatula*. They also suggest that inhibitor studies should be treated with caution because of unknown side effects of the inhibitors. The involvement of AVG and Ag^+ as a positive and negative inducer in determining the nodulation zone as presented here may provide additional tools to study the regulation of nodule development in legumes, in particular in relation to autoregulation of nodulation.

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